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The estrogen receptor (ER) regulates the expression of genes involved in the growth, proliferation and differentiation of skeletal, cardiovascular, neural and reproductive tissues. A basic scheme for the mechanism for ER action has been developed, but precise details on the interactions between ER and the cellular signaling and transcription machinery required for receptor-mediated regulation of specific target genes are still lacking. We have developed an estrogen responsive system in the fruit fly, *Drosophila melanogaster* in order to explore the functional interactions between ER and other cellular proteins. Transgenic flies carrying the human ER alpha and an estrogen responsive green fluorescent protein (GFP) reporter gene were constructed. *In vivo* expression of the GFP reporter gene was observed when larvae were grown on a food source containing steroidal or nonsteroidal estrogens. The induction of the reporter gene by estrogens was blocked upon treatment with tamoxifen, an estrogen antagonist. However, we did not recapitulate ligand-independent activation of the receptor *in vivo* or in cultured *Drosophila* cells. An estrogen responsive Drosophila system could be used to identify and characterize the complex functional interactions between ER and the other components of the cellular transcriptional apparatus.

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FOREWORD

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Vaughn C. Thackrey 6/9/00

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Introduction

Steroid receptors regulate the expression of many genes involved in human development, metabolism and homeostasis. Ectopic expression of steroid receptors in a genetically amenable organism such as the fruit fly, *Drosophila melanogaster* could provide details on the interactions between ER and the cellular signaling and transcription machinery required for receptor-mediated regulation of specific target genes. There is a significant amount of conservation of cell signaling pathways and the transcriptional apparatus between mammals and fruit flies. Not only does *Drosophila* possess homologues to many mammalian signaling proteins, chromatin remodeling factors, coregulators and basal transcription factors but *Drosophila* also express nuclear receptors. These nuclear receptors include the ecdysone receptor (EcR) which binds the steroid hormone, ecdysone, and *ultraspiracle* (*usp*), the *Drosophila* homologue to the retinoid X receptor. The fact that EcR is used as part of an inducible expression system in mammalian cells and that the glucocorticoid receptor functions in cultured *Drosophila* cells suggests that many insect and mammalian transcription factors are functionally interchangeable. We have developed an estrogen responsive system in the fruit fly, *Drosophila melanogaster* in order to explore the functional interactions between ER and other cellular proteins.

Annual Summary

Aim One: Develop an estrogen responsive fly in which a reporter gene is regulated by estrogens.

Since ecdysone and its receptor play key roles in *Drosophila* development, we wanted to test whether there was any interaction between the ER and EcR pathways. This was a potential concern since the ER homodimer and the EcR/*usp* heterodimer can recognize repeats of the same hexameric sequence although with a different orientation and spacing. The induction of an estrogen-responsive reporter gene and an ecdysone-responsive reporter gene was examined in cultured *Drosophila* cells after treatment with estrogen or ecdysone (Fig. 1). In the absence of hormone, no reporter gene activity was observed. 100 nM E_2 induced the ERE_4:lacZ reporter gene but not the $EcRE_7$:lacZ reporter gene while 1 μM 20-HE induced the $EcRE_7$:lacZ reporter gene but not the ERE_4:lacZ reporter gene. This experiment indicated that insect cells possess the necessary cellular machinery to activate transcription of an estrogen-dependent reporter gene via the human ER. These results also suggested that an ER transgene is unlikely to interfere with normal ecdysone-regulated developmental pathways and that EcR would not activate an ERE-driven transgene in flies.

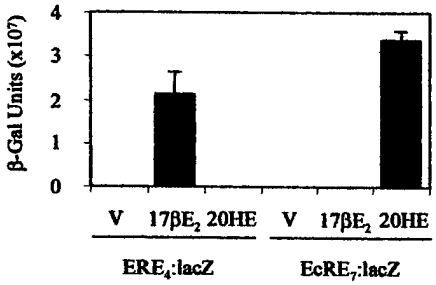


Figure 1. ER and EcR specificity in *Drosophila* cells. S2 cells containing endogenous EcR were transfected with 0.5 ug of pP{CaSpeR-actin:ER, *white*$^+$} and 0.5 ug of the ERE_4:lacZ or the $EcRE_7$:lacZ reporter gene. The cells were treated with vehicle (V), 10^{-7} M 17βE_2 or 10^{-6} M 20-HE. The data represent the mean and standard deviation of 3 experiments.

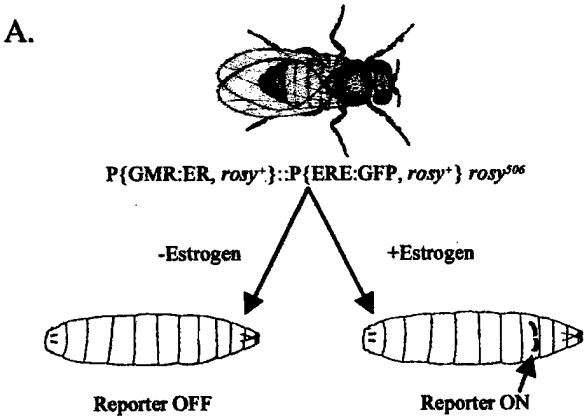
In order to create an estrogen-responsive *Drosophila*, we constructed two transgenic fly lines by P-element mediated transposition. The first contained a transposon which directed the expression of ER under the control of the glass multiple repeat. The multimerized glass element promotes transgene expression specifically in the developing *Drosophila* eye. The second line carried a GFP transgene driven by the same estrogen-responsive promoter used in the cell culture studies shown in Fig. 1. Double transgenic flies carrying ER and the GFP reporter gene on the third chromosome were obtained by recombination and recognized by GFP expression following growth on food containing 10 μM E_2.

Aim Two: Determine how estrogen receptor agonists and antagonists behave in the transgenic fly compared to mammalian cells.

The double transgenic flies carrying ER and the ERE-GFP reporter gene were assessed for estrogen responsiveness (Fig. 2A). P{GMR:ER, *rosy*$^+$}::P{ERE:GFP, *rosy*$^+$} *rosy*^{506} flies were raised on media containing hormone or vehicle control. Third instar larvae were collected and dissected to obtain eye-antennal imaginal discs. The imaginal discs were fixed at room temperature for 20 minutes in 3% formaldehyde in 3% sucrose, 1 x PBS pH 7.5. Differential interference contrast and fluorescence

photographs were taken using a Zeiss microscope at 200x magnification with Nomarski and fluorescein isothiocyanate (FITC) filters.

GFP was expressed in a hormone-responsive fashion in eye imaginal discs from third instar larvae (Fig. 2B). No reporter gene expression was observed in the absence of hormone or at 0.1μM etE_2. Expression was evident at 1μM etE_2 and increased at 10 μM etE_2. E_2 or the nonsteroidal estrogen, DES, induced GFP expression to levels comparable to etE_2. By itself, the partial agonist/antagonist, 4OHT, failed to exhibit detectable agonist activity but effectively abolished GFP expression induced by feeding on 10 μM etE_2. However, no inhibition of the response to 10 μM etE_2 was seen with 10 μM ICI, possibly because we could not use a sufficient excess of antagonist compared to agonist. In general, the *in vivo* agonist and antagonist behavior recapitulated that seen in mammalian systems. It is noteworthy that the transgenic flies exhibited a proper response to estrogen agonists and antagonists given the apparent lack of a *Drosophila* homologue to the p160 coactivator family of proteins and the weak sequence similarity of the *Drosophila* protein, SMRTER to the mammalian N-CoR/SMRT homologues.



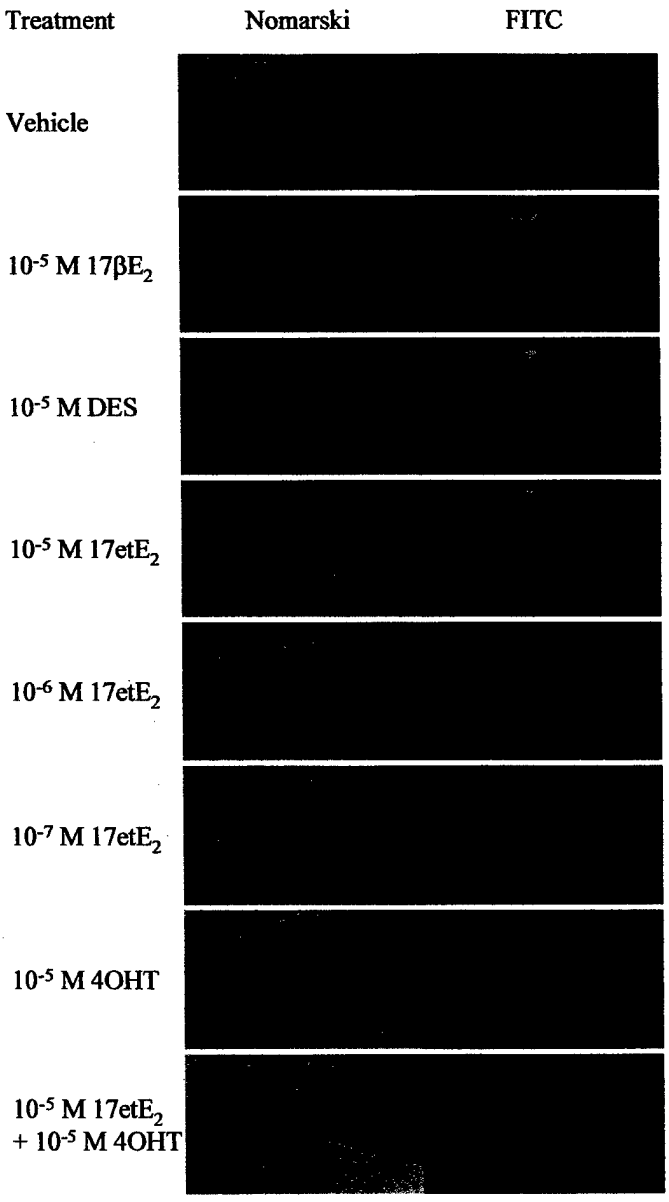


Figure 2. Estrogen responsive reporter system in *Drosophila*. A. Double transgenic flies carrying ER and the ERE-GFP reporter gene were raised on media containing vehicle or estrogen. GFP expression was assessed in the eye imaginal discs of third-instar larvae. B. Differential interference contrast and fluorescence photographs were taken at 200x magnification with Nomarski and FITC filters. Representative photographs from 2-4 experiments are shown.

One shortcoming of the present system is that we did not observe GFP expression in the adult eye although expression of a UAS-GFP reporter gene was detectable in the adult eye of flies in which eye-specific GAL4 expression is controlled by the glass multiple repeat. In addition, estrogens and estrogen antagonists were effective at doses well above those expected given the affinities of the ligands for ER. This was likely due to the fact that the ligands were taken up by ingestion from the larval medium and therefore the actual tissue concentrations of ligands were significantly lower than the concentration in the medium. In this case, lower doses of estrogen and estrogen antagonists would be expected to be effective in cultured *Drosophila* cells. This prediction was confirmed as shown in Fig. 3. Estrogen responses could be observed at subnanomolar levels of E_2, etE_2, and DES, and were maximal (>10^3 over basal) at 100nM (Fig. 3A). In cultured cells, both 4OHT and ICI antagonized induction, mediated by 10 nM E_2, in a dose-dependent fashion (Fig. 3B).

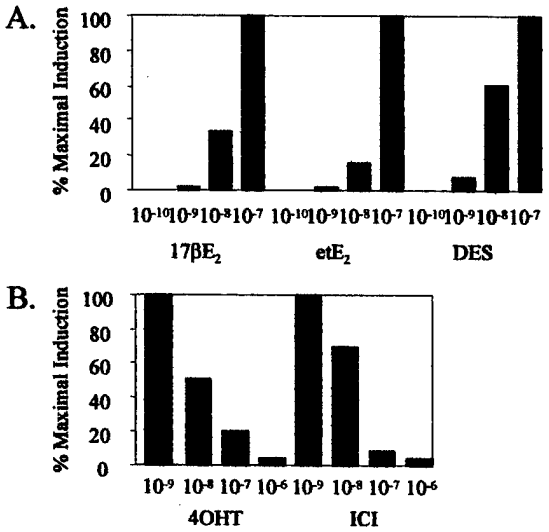


Figure 3. Dose dependent estrogen responsiveness and antagonism in cultured *Drosophila* cells. S2 cells were transiently transfected with 0.5 ug of pP{CaSpeR-actin:ER, *white*$^+$} and 5 ug of the ERE_3-TATA-luciferase reporter gene. A. The cells were treated with 10^{-10} to 10^{-7} M 17ßE$_2$, etE$_2$ or DES. B. The cells were treated with 10^{-8} M 17ßE$_2$ and 10^{-9} to 10^{-6} M 4OHT or ICI. The data represent the mean of 2-4 experiments

These experiments demonstrate that human steroid receptors can function in *Drosophila melanogaster* and suggest that *Drosophila* genetics could be applied to dissecting the mechanisms of receptor action. For example, an estrogen responsive system in *Drosophila* could be used to identify and analyze *Drosophila* cofactors that influence estrogen responsiveness. One advantage of a genetic strategy is that it would not be biased by preconceptions about the mechanism of steroid receptor action. In addition, the polytene chromosomes of *Drosophila* larval salivary glands could be used to visualize the recruitment of factors to an estrogen-responsive promoter *in vivo*.

Although ER transgenic flies imitated the mammalian response to estrogen agonists and antagonists, there was one feature of estrogen action in mammalian cells that we were unable to recapitulate. We did not observe ligand-independent activation of ER when transgenic flies carrying ER and the ERE_4-GFP were crossed with transgenic flies carrying a constitutively active *Drosophila* EGF receptor or mouse catalytic subunit of protein kinase A. Similarly, there was no activation of the ERE_4-lacZ reporter gene in S2 cells

transfected with ER following 8-bromo cAMP treatment. While the failure to observe ligand-independent ER activation does not rule out the possibility that ER could be activated in insect systems under appropriate conditions, it suggests the possibility of using complementation of the insect system to identify signaling molecules involved in ligand-independent activation.

Key Research Accomplishments

- Developed an estrogen responsive system in the fruit fly, *Drosophila melanogaster* in order to explore the functional interactions between ER and other cellular proteins.
    - Constructed transgenic flies carrying the human ER alpha and an estrogen responsive GFP reporter gene.
    - Demonstrated *in vivo* expression of the GFP reporter gene in *Drosophila* when larvae were grown on a food source containing steroidal or nonsteroidal estrogens. The induction of the reporter gene occurred in a dose dependent manner.
    - The induction of the GFP reporter gene by estrogens was blocked upon treatment with tamoxifen, an estrogen antagonist.
    - Ligand-independent activation of the receptor *in vivo* or in cultured *Drosophila* cells was not observed.

Reportable Outcomes

Poster Presentations:

Thackray, V., Young, R., Hooper, J., and Nordeen, S. (1999) A Genetic System for the Study of Steroid Hormone Action. 81st Annual Meeting of The Endocrine Society, San Diego, CA

Thackray, V., Young, R., Hooper, J., and Nordeen, S. (2000) Using a Genetic System to Study Estrogen Receptor Action. Nuclear Receptors 2000 Keystone Symposium, Steamboat, CO

Publications:

Thackray, V., Young, R., Hooper, J., and Nordeen, S. (2000) Estrogen Agonism and Antagonism on the Human Estrogen Receptor in Drosophila. (submitted)

81st Annual Meeting of the Endocrine Society, San Diego, CA
Poster Presentation

A Genetic System for the Study of Steroid Hormone Action

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The estrogen receptor regulates the growth and development of numerous tissues including those in the reproductive organs as well as the brain, bone and heart. A basic mechanism for ER action has been formulated from biochemical and functional studies. However, an unanswered question is how does ER interacts with the cellular machinery to regulate the transcription of specific target genes. We have developed an estrogen responsive genetic system in the fruitfly, *Drosophila melanogaster*, in order to address this question. Transgenic flies containing the alpha isoform of ER were crossed to transgenic flies containing an estrogen responsive reporter gene. In vivo induction of the transgene in *Drosophila* larvae was seen when 17α-ethynyl estradiol but not 17β-estradiol was introduced into the agar food source. This preliminary result indicates that *Drosophila* may recapitulate aspects of mammalian bioavailability to estrogens. The utility of two different reporter genes is being currently assessed for their use in quantitative analyses and genetic screening. An estrogen responsive *Drosophila* system holds great promise as a tool to dissect the complex functional interactions between steroid receptors and other cell signaling pathways as well as the other components of the transcriptional apparatus. The remarkable conservation of signaling pathways and the transcription machinery between flies and humans makes it highly likely that our findings will be of direct relevance to humans.

Nuclear Receptors 2000 Keystone Symposium, Steamboat Springs, CO
Poster Presentation

Using a Genetic System To Study Estrogen Receptor Action

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The estrogen receptor (ER) regulates the expression of genes involved in the growth, proliferation and differentiation of skeletal, cardiovascular, neural and reproductive tissues. A basic mechanism for the action of ER has been formulated from biochemical and functional studies but how ER interacts with the cellular machinery to regulate the transcription of specific target genes remains poorly understood. In this work, we describe the development of a genetic system, in the fruit fly, *Drosophila melanogaster*. Transgenic flies containing the alpha isoform of ER and an estrogen responsive green fluorescent protein reporter gene were constructed. Experiments in cultured insect cells indicated that the estrogen responsive reporter gene was induced by estrogen treatment. *In vivo* induction of the reporter gene was seen in *Drosophila* when estrogens were introduced into the agar food source. Interestingly, the induction of the reporter gene was blocked with treatment with an estrogen antagonist. An estrogen responsive Drosophila system holds great promise as a tool to dissect the complex functional interactions between ER and the other components of the cellular transcriptional apparatus.